A Mouse Model System to Genetically Dissect the Molecular Mechanisms Regulating Tumorigenesis

Kurt Degenhardt[1] and Eileen White[1,2]

Abstract The vast majority of human tumors are of epithelial origin and result from the accumulation of mutations that alter the function of pathways that control critical cellular processes, including proliferation, checkpoint regulation, and apoptosis. Authentically replicating these events in animal models is critical to understanding the biology of cancer and for testing the feasibility of novel therapies. We developed a mouse model that recapitulates the steps of epithelial tumor progression of multiple tissue types (kidney, breast, ovarian surface, and prostate epithelia), which takes advantage of the power of mouse genetics, and that allows for biochemical analysis, genetic selection, and screening. Moreover, this model enables functional interrogation of far more complex tumor genotypes, both of the tumor cells themselves, and of the cells in the tumor microenvironment. This is a crucial advantage, as human tumors result from multiple compound mutations, most of which are difficult to achieve through standard mutant mouse technology. We have applied this model to establish the role of apoptosis in epithelial solid tumor progression and in treatment response, which has provided novel opportunities for cancer therapies in humans.

Mouse models have long played an essential role in biological research in multiple disciplines from development to tumor biology. Mutation of a specific gene or genes in the mouse in the relevant tissue at the appropriate time in development can authentically replicate many human disease states, providing strong evidence for the role of specific genes in the disease process. This approach has been invaluable for understanding the molecular basis of innumerable human pathologic conditions. The ability to recapitulate human disease in a mouse model has also been instrumental in revealing and testing possible therapies. Although there are clear physiologic and genetic differences between mice and man, mice are a fair representation of human biology that can be manipulated genetically. Furthermore, many if not all of the molecular pathways implicated and tumorigenesis in humans are conserved in mice. Genetic recapitulation of a human tumor phenotype in the mouse provides proof of principle for the role of a given gene or pathway in oncogenesis, a biological system with which to study the disease process, and a platform for assessing the usefulness of potential therapies. Through these studies, we have identified a p53-independent apoptotic pathway for suppressing tumorigenesis *in vivo* that is signaled through the BH3-only proapoptotic protein BIM, which requires proapoptotic BAX or BAK and which is inactivated by a gain of function of antiapoptotic BCL-2. Furthermore, this pathway is targeted for inactivation by common mutations found in human tumors that up-regulate the activity of the mitogen-activated protein kinase pathway, thereby promoting tumor progression and resistance to chemotherapeutics through direct inactivation of BIM. These findings establish that determining the molecular means by which tumors defeat apoptosis can lead to a rational, tumor genotype-specific approach to chemotherapy. Applying this technology to other poorly understood processes implicated in the regulation of tumorigenesis, such as autophagy, may be similarly beneficial.

Generation of Immortal Mouse Epithelial Cells: The Immortal Baby Mouse Kidney Epithelial Cell Model

As epithelial cells are the source of most human tumors, studying their normal biology *in vivo* and *in vitro* and how their epithelial properties are altered during tumorigenic progression is important. Unlike other cell types, such as fibroblasts, primary epithelial cells can be much more challenging to isolate and culture *in vitro*, which often limits the amount of material available for study. Epithelial cell lines derived from human and mouse tumors, however, are in abundant supply and have been used extensively in research. Although much has been learned by studying human cancer cell lines, it is more advantageous to have a system whereby the stepwise progression of normal epithelial cells to their tumorigenic derivatives can be examined. Cell lines derived from human tumors have an undefined and complex mutational history from which it is often difficult to decipher the molecular events that lead to their creation. Furthermore, *in vivo* tumorigenesis is restricted to tumor

Authors' Affiliations: [1]Center for Advanced Biotechnology and Medicine, Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, New Jersey and [2]Cancer Institute of New Jersey, New Brunswick, New Jersey
Received 2/23/06; revised 6/14/06; accepted 6/22/06.
Requests for reprints: Eileen White, Center for Advanced Biotechnology and Medicine, Department of Molecular Biology and Biochemistry, Rutgers University, 679 Hoes Lane, Piscataway, NJ 08854. Phone: 732-235-5329; Fax: 732-235-5795; E-mail: ewhite@cabm.rutgers.edu.

doi:10.1158/1078-0432.CCR-06-0439

xenografts in immunocompromised mice in a nonphysiologic environment. Our alternative approach has been to isolate and culture normal mouse primary epithelial cells and to develop the genetic means to immortalize them such that they retain their normal epithelial characteristics. This enables production of genetically defined mouse epithelial cell lines in sufficient quantities for long-term analysis. As these immortal epithelial cell lines are nontumorigenic, they provide a beneficial starting point for investigating the molecular and biological events that promote tumorigenesis in a completely genetically defined system in the physiologically relevant host. This model takes advantage of powerful genetically engineered mouse models yet is complementary by facilitating more genetic and biochemical analysis that may otherwise be difficult without access to cells for analysis and manipulation *in vitro* (Fig. 1).

Early studies have shown that cellular immortalization requires disruption of two ubiquitous cellular pathways for tumor suppression: the retinoblastoma (RB) pathway that regulates cell cycle progression through the G_1-S restriction point and the p53 pathway that regulates cell cycle checkpoint control in response to cellular damage and stress (1, 2). The RB pathway can be deregulated by loss of *rb* or deregulation of individual effectors of the pathway upstream or downstream of RB. Alternatively, viral oncoproteins, such as adenovirus E1A, human papillomavirus E7, or SV40 T antigen, similarly inactivate the RB pathway but do so by binding directly to RB, thereby providing a convenient experimental tool for driving epithelial cell proliferation (1, 3, 4). Inactivation of the RB pathway, however, activates p53 to induce either growth arrest or apoptosis, which necessitates concurrent RB and p53 inactivation for epithelial immortalization (1, 2). Inactivation of the p53 pathway can be achieved through loss of p53, expression of a p53 mutant proteins that typically act in a dominant-negative fashion, or through expression of the viral oncoproteins adenovirus E1B 55K, human papillomavirus E6, or SV40 T antigen, which bind and directly inactivate p53. Thus, numerous genetic tools exist for immortalizing epithelial cells, although human epithelial cells additionally require activation of telomerase to bypass life span limitations caused by telomere erosion (5).

We have found that expression of E1A or the cellular oncoprotein c-Myc with the dominant-negative p53 mutant p53DD, which encodes the p53 oligomerization domain, is necessary and sufficient for immortalization of primary baby rat kidney and baby mouse kidney (BMK) epithelial cells as well as epithelial cells of multiple tissue types (6–14). As immortalization of primary epithelial cells is a selection for function (immortal cells forming a colony), expression of selectable drug markers typically used to generate cell lines expressing specific genes from plasmids is not necessary. These immortal BMK epithelial cells (iBMK; refs. 7, 14–17), mouse mammary epithelial cells (iMMEC), mouse ovarian surface epithelial cells, and mouse prostate epithelial cells retain their normal epithelial characteristics yet are nontumorigenic (Fig. 1).

Adaptation of the iBMK Model to Study Ovarian, Breast, and Prostate Cancers

Cancer is not a single disease but rather many different diseases with characteristics often relating to the tissue of origin. These tissue-specific distinctions in cancer arise from differential intrinsic characteristics of individual cell types, the propensity and susceptibility to particular oncogenic mutations of that cell type, and variations in the tissue microenvironment. To begin to address tissue-specific issues in cancer progression, we immortalized not only kidney but also breast, ovarian surface, and prostate epithelial cells with E1A and p53DD to model cancer progression in those tissues as well (Fig. 1).

iMMECs have been derived from primary mouse mammary epithelia and, as found with iBMK cells, are nontumorigenic, and as in human breast cancer, tumorigenicity is conferred by activation of HER-2/*neu*. A unique attribute of iMMECs is their ability to undergo three-dimensional morphogenesis in basement membrane cultures where they form spherical acini that create hollow lumens through apoptosis,[3] similarly to human MCF-10A cells (18–21). This ability of iMMECs to mimic epithelial glandular morphogenesis *in vitro*, a process that is highly disturbed by oncogenic mutations in breast cancer, will provide another context with which to interrogate the process of oncogenesis. Three significant advantages of iMMECs over the MCF-10A system are that iMMECs are genetically defined, can take advantage of the availability of mutant mice, and, because they are C57B/6 derived, tumorigenesis can be evaluated in an immunocompetent host. Another advantage includes the ability to do tumor growth in the physiologic microenvironment of the mouse mammary gland through orthotopic injection into the mammary fat pad. iMMECs derived from mutant mice with deficiencies in apoptotic regulators and autophagy (*beclin1*$^{+/-}$) are currently under investigation.

Human ovarian cancer is a devastating disease, and models to study it are limited. We have isolated and immortalized primary immortalized mouse ovarian surface epithelial cells, which are nontumorigenic on injection into the peritoneal cavity, the physiologic microenvironment for growth of this type of cancer.[4] Tumorigenicity of immortalized mouse ovarian surface epithelial cells is conferred by activation of the mitogen-activated protein kinase pathway or blockade of apoptosis, the later of which forms tumors with a phenotype that resembles papillary serous type of stages III and IV ovarian carcinomatosis. Numerous tumors develop throughout the peritoneal cavity with marked tropism to ovaries, omentum, intestinal mesentery, and diaphragm, often with ascites fluid accumulation as in the human disease. Having this mouse model system that mimics ovarian carcinomatosis allows us to address issues unique to ovarian cancer patients. Similar questions related to the biology of prostate cancer are being addressed with immortalized mouse prostate epithelial cells.

Application of Mutant Mice to Probe Gene Function in Epithelial Immortalization

Targeted gene disruption methods have led to the creation of mutant mice, and one intended purpose of the development of our immortalized mouse epithelial cell systems was to apply

[3] V. Karantza-Wadsworth and E. White, in preparation.
[4] C. Karp, W. Nieves-Neira, and E. White, in preparation.

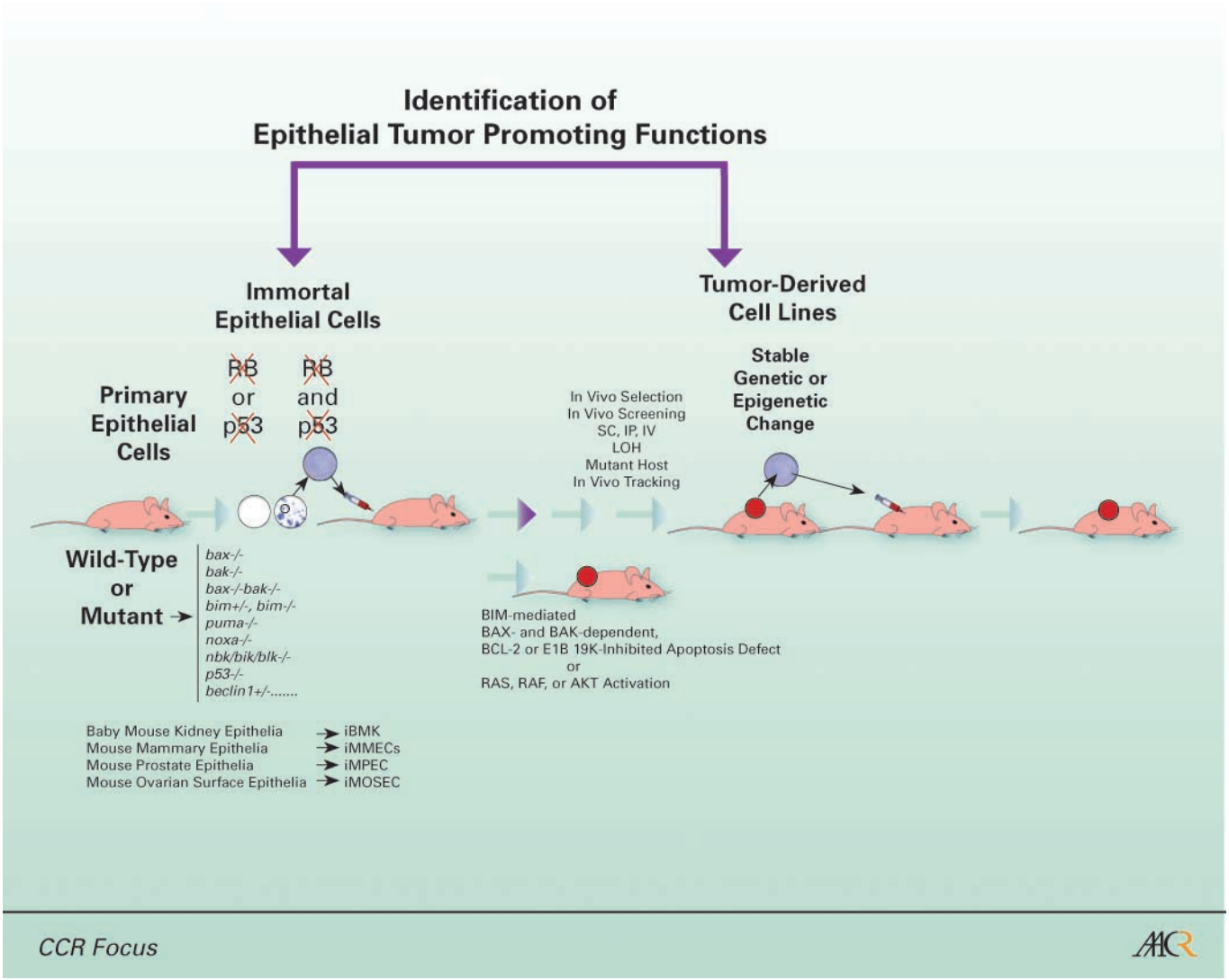

Fig. 1. The mouse epithelial immortalization model for the identification of tumor-promoting functions. Primary epithelial cells are isolated from wild-type (C57B/6) BMK (ages 0-8 days; ref. 7) or from adult (after pubescent) mouse breast, prostate, or ovarian surface epithelia. The isolated primary epithelia are transfected by electroporation with expression vectors for E1A or c-Myc and p53DD, or alternatively, epithelia from $p53^{-/-}$ mice are transfected with E1A alone. Following 1 week of *in vitro* culture, untransfected epithelial cells die off leaving behind immortal epithelial colonies that emerge in the next 2 to 3 weeks without the requirement for drug selection. Immortalized colonies are then cloned and expanded, and all express the input oncogenes as there is no spontaneous epithelial cell immortalization in this assay. Immortal epithelial cell lines have been derived from different mouse epithelial tissues, including kidney (*iBMK*), breast (*iMMECs*), ovary (*iMOSECs*), and prostate (*iMPECs*). On injection into athymic nude mice (*nu/nu*), these immortal epithelial cells are clonally tumorigenic: approximately 1 in 10^6 cells is capable of tumorigenic growth (14, 16); when challenged for tumorigenicity in an immunocompetent, syngeneic host (C57B/6), the frequency is further reduced. Tumorigenesis can also be evaluated for growth by varying the genotype of the recipient host mouse. Those selected clones that do form tumors (tumor-derived cell lines) have acquired a stable genetic or epigenetic change manifested by specific alterations in gene expression and acquisition of phenotypic changes that confer tumorigenic growth. This *in vivo* selection for tumorigenesis is also the basis for genetic screening using retroviral libraries (both cDNA and short hairpin RNA) for genes, the gain or loss of function of which contributes to tumor growth. Tumor growth can be interrogated s.c., orthotopically in the mammary fat pad (*iMMEC*) or prostate (*iMPECs*), i.p. (*iMOSEC*), and i.v. to evaluate metastatic potential. Growth of immortalized epithelial cells can be followed *in vivo* noninvasively by engineering the cells to express red or green fluorescent protein (Lighttools optical imaging) or luciferase (IVIS chemiluminescent imaging; ref. 16). To test the role of specific genes in tumorigenesis, immortalized epithelial cells are derived from mutant mice ($p53^{-/-}$, $bax^{-/-}$, $bak^{-/-}$, $bax^{-/-}bak^{-/-}$, $bim^{-/-}$, $puma^{-/-}$, $noxa^{-/-}$, $nbk/bik/blk^{-/-}$, and $beclin1^{+/-}$). iBMK cells with deficiencies in BIM or BAX and BAK that were derived from the corresponding mutant mice are defective for apoptosis and acquire tumorigenicity (14, 17). iBMK cells that retain an intact apoptotic response conferred by a single wild-type *bax* or *bak* allele undergo loss of heterozygosity (*LOH*) during selection *in vivo* for tumorigenesis that results in deficiency in BAX and BAK and resistance to apoptosis (14). Thus, defects in apoptosis are selected for *in vivo* during tumor evolution. Similarly, BCL-2 expression blocks apoptosis and promotes tumorigenesis and expression of the BAX and BAK inhibitor and viral BCL-2 homologue, E1B 19K, blocks apoptosis, and enables tumorigenic growth (16). Neither BCL-2 nor E1B 19K expression has any measurable tumor-promoting activity in the absence of BAX and BAK showing epistasis, indicating that they function in the BAX and BAK pathway to promote tumorigenesis (16). Expression of activated H-RAS, RAF, and AKT also overcome the tumorigenic block of wild-type iBMK cells, and H-RAS and RAF do this in part through inactivation of BIM (17). Finally, allelic loss of the essential autophagy gene *beclin1* in iBMK cells confers sensitivity to metabolic stress and promotes tumor formation (15).

this technology to this large pool of mutant mice to address key questions in epithelial tumorigenesis. The first test of the feasibility of this approach used mice deficient for the tumor suppression gene *p53*. There was strong evidence that p53 inactivation was required for immortalization of BMK cells by E1A or c-Myc expression, and the ultimate test was that E1A alone should be sufficient for immortalizing BMK cells from p53-deficient mice. As predicted, primary BMK cells from

p53-deficient mice are immortalized solely by E1A, whereas immortalization of primary wild-type BMK cells requires coexpression of both E1A and p53DD (7). This result illustrates that the immortalization activity of p53DD is caused by its functional inactivation of p53 (7).

Neither the $p53^{-/-}$ iBMK cells nor those immortalized by E1A and p53DD are tumorigenic when injected s.c. into nude mice, suggesting that additional functions beyond RB and p53 pathway inactivation are required for solid tumor formation (14). As these iBMK cells retain an intact apoptotic response, this suggests that inactivation of p53-independent apoptosis may be additionally required for tumorigenesis. Many genes that regulate apoptosis have been mutated in mice, and several of them are linked to lymphoma development (22); however, their role in solid tumor formation was not known. We took a systematic approach to assessing the role of key apoptosis regulators in epithelial tumorigenesis by generating iBMK cells from mutant mice with targeted gene mutations in the apoptotic pathway and evaluating their tumorigenic potential.

The Apoptotic Signaling Pathway

As discoverers of a viral oncogene that encodes an antiapoptotic function, the adenovirus *E1B 19K* gene (2, 23), we are interested in the role that apoptosis plays in suppressing tumorigenesis and facilitating successful cancer treatment. Apoptosis or type I programmed cell death is an evolutionally conserved process that eliminates individual cells through suicide or murder (24). Apoptosis occurs in response to developmental cues and disease states, serves to sculpt tissues, eradicates abnormal or infected cells without inflammation, and is required for maintenance of homeostasis. BCL-2 family proteins are central regulators of apoptosis signaled through mitochondria. Multidomain BCL-2 family members function either to inhibit (e.g., BCL-2 and MCL-1) or to promote (e.g., BAX and BAK) apoptosis. BH3-only BCL-2 family members (e.g., BIM, BID, NBK/BIK/BLK, PUMA, and NOXA) are all proapoptotic and serve either to antagonize the survival activity of BCL-2-like proteins or to activate the proapoptotic function of BAX and BAK (25–27). The coordinate inhibition of BCL-2-like proteins and activation of BAX and BAK is controlled by the binding specificity of BH3-only proteins to their targets (25, 27). BIM is a specific antagonist of BCL-2 (28), whereas NOXA disrupts MCL-1 from binding and inhibiting BAK (29, 30), and BID is a BAX and BAK activator (31, 32). Once activated, BAX and BAK may form pores and/or oligomerize in the mitochondrial outer membrane, rendering it permeable to proapoptotic mitochondrial proteins, such as cytochrome *c* and SMAC/DIABLO (33). Once released into the cytoplasm, cytochrome *c* activates the cysteine protease caspase-9 in the apoptosome, whereas SMAC/DIABLO antagonizes caspase inhibitors (inhibitor of apoptosis proteins). Subsequent effector caspase activation (e.g., caspase-3) leads to the orderly dismantling of the cell without inflammation. There is ample evidence that numerous components of this apoptotic pathway are deregulated in human tumors and that effective treatment may rely on successful activation of apoptosis. Although many of the genes that regulate apoptosis have been mutated in mice, deciphering their potential role in epithelial cancers in most cases is problematic. This is due to developmental phenotypes that preclude viability for the length of time required for tumor development and/or the necessity of crossing the mutations into tumor-prone genetic backgrounds, the complexity of which is compounded by functional redundancy.

Use of the iBMK Model to Show the Tumor Suppressor Activity of BAX and BAK

BAX and BAK have substantially overlapping roles in the implementation of apoptosis. Deficiency in either one alone in the mouse results either in a mild or in an absent phenotype, whereas deficiency in both profoundly abrogates apoptosis and results in neonatal lethality (34). To test the requirement for BAX- and BAK-mediated apoptosis in cellular immortalization and tumorigenesis, iBMK cells were generated from the singly and doubly deficient mice. Failure of immortalization with E1A alone showed that the absence of BAX and/or BAK did not alleviate the requirement for inactivating p53. As p53 induces growth arrest and apoptosis, both of which have been linked to its tumor suppressor activity (35), blocking only apoptosis may not be sufficient to prevent cellular immortalization. Indeed, p53 induces a potent growth arrest response in cells where p53-mediated apoptosis is blocked by BCL-2 or E1B 19K expression (12, 36). As expected, the combination of E1A and p53DD immortalizes BMK cells from the BAX and BAK singly and doubly deficient mice, and only BAX and BAK-deficient iBMK cells are refractory to apoptosis induction (Fig. 1; ref. 7).

These iBMK cell lines are not only genetically defined but also isogenic because iBMK cells from mutant mice are derived in parallel with iBMK from heterozygous and wild-type littermates. Importantly, this method of generating immortal epithelial cell lines can be adapted to interrogating the function of any gene in which the mutant mouse survives to near birth and provides an alternative to mouse embryo fibroblasts that is more cancer relevant. Although we have focused our efforts initially on mice with deficiencies in members of the BCL-2 family, this method is not limited to genes involved in apoptosis. We have recently used this approach to decipher the role of autophagy in epithelial tumorigenesis through the generation and characterization of iBMK cells from *beclin1*$^{+/-}$ mice (Fig. 1; ref. 15).

Benefits of Immortalized Epithelial Cells for Functional Analysis *In vitro*

Apoptosis is prevented by the retention of cytochrome *c* and SMAC/DIABLO in the intermembrane space of mitochondria in healthy cells and is activated by their release from mitochondria into the cytosol. When challenged with an apoptotic stimulus, wild-type, BAX, or BAK-deficient iBMK cells efficiently release cytochrome *c* and SMAC/DIABLO from mitochondrial stores into the cytoplasm, which causes activation of caspase-9 and caspase-3 and cell death by apoptosis. When similarly challenged, BAX and BAK-deficient iBMK cells retain cytochrome *c* and SMAC/DIABLO within mitochondria, fail to activate caspases, and remain viable (7). The availability of genetically defined cells in conjunction with cellular and biochemical analysis *in vitro* was essential for doing this type of analysis. This pivotal requirement for BAX or BAK to implement the release of

apoptogenic proteins from intracellular stores to promote apoptosis is consistent with the phenotype of the mutant mice and cells derived there from (34, 37).

One confounding factor in the field of cell death is sorting out cause and effect. The very process of cell death affects nearly every aspect of cellular function, only some of which are instrumental in the execution of cell death. Having the live cells, however, allows all of the upstream events in apoptosis to be examined unfettered by the downstream death process. This approach revealed the role of proapoptotic BH3-only proteins upstream of BAX and BAK and the manifestation of chromosome instability in response to metabolic stress conferred by defective apoptosis (16, 38). Genetically defined, apoptosis-deficient iBMKs have also been useful for sorting out the contribution of apoptosis to a wide variety of cellular processes from RNA processing to the antiviral response (39, 40).

iBMK Cells Reveal a p53-Independent Pathway for Suppression of Tumorigenesis

As wild-type iBMK cells, which retain an intact apoptotic response, are not tumorigenic, the question arose about the tumorigenicity of the apoptosis-defective BAX and BAK-deficient iBMK cells. In s.c. nude mouse allografts, BAX/BAK-deficient iBMK cells form highly invasive carcinomas. In contrast, iBMKs that express BAX and BAK are poorly tumorigenic, forming tumors only after 3 to 4 months that result from clonal emergence (14, 16). These observations identified a novel p53-independent apoptosis pathway for tumor suppression, identified BAX and BAK as tumor suppressor proteins, and supported the observed loss of BAX and/or BAK in human tumors as a contributing factor in tumor progression (Fig. 1; ref. 14).

Selection *In vivo* for Loss of Heterozygosity

Remarkably, iBMK cells with $bax^{-/-}bak^{+/-}$ or $bax^{+/-}bak^{-/-}$ genotypes eventually form tumors, but nearly all tumors undergo loss of heterozygosity for the remaining *bax* or *bak* allele generating tumors with the $bax^{-/-}bak^{-/-}$ genotype imparting apoptosis resistance and acquisition of tumorigenicity (14). Thus, selection *in vivo* for tumorigenesis coordinately selects for defects in apoptosis, revealing the usefulness of loss of heterozygosity as an additional genetic tool for evaluating tumorigenic functions in the iBMK model (Fig. 1). As shown by the allelic loss of *bax* and *bak*, if loss of function of a given gene provides a selective advantage that imparts enhanced tumor growth, iBMK cells generated from heterozygous mutant mice and selection for loss of heterozygosity *in vivo* can be used as an unbiased functional test (Fig. 1). This attribute of the model may be particularly useful where a null mutant mouse phenotype produces a developmental phenotype that precludes evaluation of a role in promoting tumor growth.

Demonstration of Genetic Epistasis

BCL-2 is overexpressed human follicular in B-cell lymphoma due to chromosomal translocation and represents an example of a gain-of-function apoptosis defect that contributes to oncogenesis. (22). BCL-2 expression in wild-type iBMK cells also confers apoptosis resistance *in vitro* and tumorigenicity *in vivo*, thereby recapitulating the phenotype of the BAX and BAK-deficient iBMK cells (16). More importantly, BCL-2 expression in BAX and BAK-deficient iBMK cells has no detectable additional activity *in vitro* or *in vivo*, clearly showing that BCL-2 functions in the BAX and BAK pathway to block apoptosis and promote tumor growth (16). Similarly, expression of the direct BAX and BAK inhibitor, E1B 19K, blocks apoptosis and promotes tumorigenesis of wild-type iBMK cells but has no measurable activity in the absence of BAX and BAK (16). These elegant demonstrations of genetic epistasis would be otherwise difficult to achieve without the ability to combine multiple gene alterations rendered possible by genetically defined iBMK cells. Clear demonstration that BCL-2 function is entirely dependent, either directly or indirectly, on inhibition of BAX and BAK for promoting epithelial tumorigenesis is an important concept in delineating the signaling pathway for cell death.

iBMK Cells Reveal a Role for BIM in the Suppression of Epithelial Tumorigenesis

Upstream of BAX, BAK, and BCL-2 are the most apical members of the BCL-2 family, the BH3-only proteins, some of which are predicted to function as tumor suppressors. Many of the genes encoding BH3-only proteins have been mutated in mice, which indicated a role for some in suppressing growth of hematopoietic malignancies but not solid tumors. This may be only due to differences in the mutational and kinetic requirements for generating carcinomas. To test the role of BH3-only protein suppression of epithelial tumorigenesis, we generated iBMK cells from *bim*, *puma*, *noxa*, and *nbk/bik/blk* mutant mice (Fig. 1) and evaluated their tumorigenic potential. Only iBMK cells deficient from BIM are tumorigenic, which showed a role for BIM as an epithelial tumor suppressor (17). In support of the tumor suppressor function of BIM, alterations, primarily deletions, in the human *bim* gene on chromosome 2q13 have been reported in human malignancies, with more than half of the cases being epithelial in origin. It remains possible that PUMA, NOXA, and NBK may function as tumor suppressors in other circumstances. PUMA and NOXA, for example, are known downstream effectors of p53 (25–27), the activity of which would not be apparent in our analysis. Nonetheless, the BH3-only deficient iBMK cell lines will continue to be useful in discerning the role of this important subclass of BCL-2 family members in apoptosis.

Use of the iBMK Model to Show Tumor Genotype-Based Rational Chemotherapy

Most chemotherapeutic drugs currently in use can function in all or in part through induction of apoptosis, but how apoptosis comes about is not entirely clear. We reasoned that determining the mechanism of chemotherapy-mediated apoptosis and the means by which it is defeated by common mutations in human tumors would provide novel opportunities to therapeutically reconstitute functional apoptosis. To this end, we embarked on a genetic approach that takes advantage of the iBMK cells with defined genetic alterations in specific components of the apoptotic signaling pathway. Focusing on the mechanism of apoptosis induction by the taxane class of antimicrotubule chemotherapeutic drugs, we discovered that

paclitaxel induces the robust accumulation of BIM, and BIM-deficient iBMK cells are specifically paclitaxel resistant (17). A repertoire of iBMK cells with defined deficiencies in key apoptosis regulators to survey rendered it possible to readily identify BIM as a determinant in taxane responsiveness *in vitro* and *in vivo*. The mechanism by which other classes of chemotherapeutic drugs regulate apoptosis is currently under investigation.

How common mutations in human tumors inactivate apoptosis was revealed by iBMK cells expressing activated H-RAS and RAF, which cause the extracellular signal-regulated kinase–mediated phosphorylation and proteasome-mediated degradation of BIM. H-RAS or RAF activation prevents BIM accumulation in response to paclitaxel producing drug resistance (17). A genetic epistasis test (H-RAS or RAF activation in BIM wild-type and deficient iBMK cells) revealed that inactivation of BIM by the mitogen-activated protein kinase pathway is responsible for defective apoptosis and taxane resistance *in vitro* and *in vivo* (17). The ability to combine multiple gain-of-function and loss-of-function mutations in this isogenic, genetically defined system made possible what would otherwise be extremely difficult experiments.

Ultimately, the intention is to use mechanistic information gleaned from molecular analysis of apoptosis regulation and to translate these discoveries into effective therapies in humans. As a first step toward this goal, we tested the prediction that resurrection of BIM function, by blocking its degradation by the proteasome pathway in tumor cells where the mitogen-activated protein kinase pathway is activated, would restore the therapeutic response to taxanes. Indeed, tumors with activated H-RAS are rendered taxane responsive by the proteasome inhibitor bortezomib (Velcade) in a BIM-dependent fashion (17). Activation of the mitogen-activated protein kinase pathway is a common property among the human tumors most refractory to treatment, and these findings provide a rational, tumor genotype-specific basis for therapy. As our knowledge of the molecular events leading to cancer continues to expand, moving from the empirical toward personalized medicine, where a specific genotype can be linked to treatment, is a desirable goal. This holds the most promise for disease management and cure in the future (41). Facile animal models, such as that described here, which can replicate the complexity of human disease, especially those amenable to preclinical testing, are a means to help achieve this goal.

In vivo Selection and Screening for Genes That Regulate Tumorigenesis

A powerful application of the immortalized but nontumorigenic epithelial cell lines we have developed is the ability to select and screen *in vivo* for genetic or epigenetic events that enable tumor growth (Fig. 1). Wild-type iBMK cells are clonally tumorigenic with approximately one in a million cells evolving to be capable of tumor growth over the course of selection for several months *in vivo* (16). Cells isolated from the resulting tumors readily return to *in vitro* culture without selection, and when challenged to form tumors again, tumorigenesis is greatly accelerated. These tumor-derived cell lines have acquired a stable genetic or epigenetic change that enables tumor growth through selection *in vivo*.

Direct comparisons between the parental wild-type and tumor-derived cell lines are providing insight into the spectrum of functions that facilitate epithelial carcinogenesis. Phenotypic characterization combined with gene expression profiling is indicating that specific, identifiable functions are acquired through selection *in vivo*. This approach is distinct from that similarly applied to human tumors where unambiguous interpretation of microarray and functional data is more problematic. The large number of changes that occur in human tumors and surrounding stroma over many years and the inherent tumor heterogeneity often confound interpretation of this type of analysis. In contrast, the tumor-derived cell lines we have generated are immortal to begin with, and we have determined that a single or small number of changes are sufficient for tumorigenesis. Indeed, microarray analysis of parental and tumor-derived cell lines confirms the specific nature of the changes that occur on selection for tumorigenesis *in vivo*. Furthermore, the nontumorigenic parent can be compared with the tumorigenic derivatives *in vitro*, separating potential complications due to stromal contributions and the microenvironment. This gene discovery approach has the potential to reveal novel functions that contribute to tumor progression.

In a complementary approach, cDNA and short hairpin RNA retroviral libraries are being introduced into wild-type iBMK cells followed by selection *in vivo* for tumorigenesis to screen for genes where a gain or loss of function will promote tumorigenesis (Fig. 1). How tissue specificity, differences in the genetic background, and selection for growth in different microenvironments alter the requirements for tumorigenicity will be of great interest. Collectively, these approaches should reveal the constellation of genetic and phenotypic events that contribute to solid tumor formation. Eventually, these strategies will be adapted to address mechanisms governing on therapeutic responsiveness and the effect of the mode of cell death on tumorigenesis (42). Although this mouse model system was originally designed to probe the involvement of apoptosis in tumorigenesis, it has evolved into a very powerful model to explore the effect of both tumor microenvironment and epithelial tumor cells themselves in tumorigenesis beyond apoptosis. Most recently, using iBMK cells derived from allelic loss of the essential autophagy gene *beclin1*, we have shown that the catabolic process of autophagy is a survival pathway used by epithelial tumor cells to survive metabolic stress, the insights from which have revealed novel opportunities for cancer treatment (15).

References

1. White E. Regulation of the cell cycle and apoptosis by the oncogenes of adenovirus. Oncogene 2001;20:7836–46.
2. White E. Mechanisms of apoptosis regulation by viral oncogenes in infection and tumorigenesis. Cell Death Differ 2006;13:1371–7.
3. Berk AJ. Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus. Oncogene 2005;24:7673–85.
4. Helt AM, Galloway DA. Mechanisms by which DNA tumor virus oncoproteins target the Rb family of pocket proteins. Carcinogenesis 2003;24:159–69.
5. Hahn W, Weinberg R. Rules for making human tumor cells. N Engl J Med 2002;347:1593–603.
6. Debbas M, White E. Wild-type p53 mediates apoptosis by E1A which is inhibited by E1B. Genes Dev 1993;7:546–54.
7. Degenhardt K, Sundararajan R, Lindsten T, Thompson CB, White E. Bax and Bak independently promote cytochrome *c* release from mitochondria. J Biol Chem 2002;277:14127–34.

8. Han J, Sabbatini P, Perez D, Rao L, Modha D, White E. The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. Genes Dev 1996;10:461–77.
9. Lin H-JL, Eivner V, Prendergast GC, White E. Activated H-ras rescues E1A-induced apoptosis and cooperates with E1A to overcome p53-dependent growth arrest. Mol Cell Biol 1995;15:4536–44.
10. Sabbatini P, Chiou S-K, Rao L, White E. Modulation of p53-mediated transcriptional repression and apoptosis by the adenovirus E1B 19K protein. Mol Cell Biol 1995;15:1060–70.
11. Sabbatini P, Han JH, Chiou S-K, Nicholson D, White E. Interleukin 1 β converting enzyme-like proteases are essential for p53-mediated transcriptionally dependent apoptosis. Cell Growth Differ 1997;8:643–53.
12. Sabbatini P, Lin J, Levine AJ, White E. Essential role for p53-mediated transcription in E1A-induced apoptosis. Genes Dev 1995;9:2184–92.
13. Sakamuro D, Eviner V, Elliot KJ, Showe L, White E, Prendergast GC. c-Myc induces apoptosis in epithelial cells by both p53-dependent and p53-independent mechanisms. Oncogene 1995;11:2411–8.
14. Degenhardt K, Chen G, Lindsten T, White E. Bax and Bak mediate p53-independent suppression of tumorigenesis. Cancer Cell 2002;2:193–203.
15. Degenhardt K, Mathew R, Beaudoin B, et al. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 2006;10:51–64.
16. Nelson D, Tan T-T, Rabson AB, Anderson D, Degenhardt K, White E. Hypoxia and defective apoptosis drive genomic instability and tumorigenesis. Genes Dev 2004;18:2095–107.
17. Tan T-T, Degenhardt K, Nelson DA, et al. Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. Cancer Cell 2005;7:227–38.
18. Debnath J, Brugge JS. Modelling glandular epithelial cancers in three-dimensional cultures. Nat Rev Cancer 2005;5:675–88.
19. Debnath J, Mills KR, Collins NL, Reginato MJ, Muthuswamy SK, Brugge JS. The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. Cell 2002;111:29–40.
20. Debnath J, Muthuswamy S, Brugge J. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods 2003;30:256–68.
21. Weaver V, Bissell M. Functional culture models to study mechanisms governing apoptosis in normal and malignant mammary epithelial cells. J Mammary Gland Biol Neoplasia 1999;4:193–201.
22. Cory S, Huang DC, Adams JM. The Bcl-2 family: roles in cell survival and oncogenesis. Oncogene 2003;22:8590–607.
23. Cuconati A, White E. Viral homologues of Bcl-2: role of apoptosis in the regulation of virus infection. Genes Dev 2002;16:2465–78.
24. Adams JM. Ways of dying: multiple pathways to apoptosis. Genes Dev 2003;17:2481–95.
25. Gelinas C, White E. BH3-only proteins in control: specificity regulates MCL-1 and BAK-mediated apoptosis. Genes Dev 2005;19:1263–8.
26. Puthalakath H, Strasser A. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ 2002;9:505–12.
27. Willis SN, Adams JM. Life in the balance: how BH3-only proteins induce apoptosis. Curr Opin Cell Biol 2005;17:1–9.
28. Bouillet P, Cory S, Zhang LC, Strasser A, Adams JM. Degenerative disorders caused by Bcl-2 deficiency prevented by loss of its BH3-only antagonist Bim. Dev Cell 2001;1:645–53.
29. Cuconati A, Mukherjee C, Perez D, White E. DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. Genes Dev 2003;17:2922–32.
30. Willis SN, Chen L, Dewson G, et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev 2005;19:1294–305.
31. Perez D, White E. TNF-α signals apoptosis through a Bid-dependent conformational change in Bax that is inhibited by E1B 19K. Mol Cell 2000;6:53–63.
32. Wei MC, Lindsten T, Mootha VK, et al. tBid, a membrane-targeted death ligand, oligomerizes Bak to release cytochrome *c*. Genes Dev 2000;14:2060–71.
33. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science 2004;305:626–7.
34. Lindsten T, Ross AJ, King A, et al. The combined functions of the pro-apoptotic Bcl-2 family members, Bak and Bax, are essential for the normal development of multiple tissues. Mol Cell 2000;6:1389–99.
35. Vousden KH, Prives C. P53 and prognosis: new insights and further complexity. Cell 2005;120:7–10.
36. Chiou S-K, Rao L, White E. Bcl-2 blocks p53-dependent apoptosis. Mol Cell Biol 1994;14:2556–63.
37. Wei M, Zong W-X, Cheng E, et al. Proapoptotic Bax and Bak: a requisite gateway to mitochondrial dysfunction and death. Science 2001;292:727–30.
38. Zong W-X, Lindsten T, Ross AJ, MacGregor GR, Thompson CB. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev 2001;15:1481–6.
39. Schwerk C, Prasad J, Degenhardt K, et al. ASAP, a novel protein complex involved in RNA processing and apoptosis. Mol Cell Biol 2003;23:2981–90.
40. Cuconati A, Degenhardt K, Sundararajan R, Anschel A, White E. BAK and BAX function to limit adenovirus replication through apoptosis induction. J Virol 2002;76:4547–58.
41. Tan T-T, White E. Therapeutic targeting of death pathways in cancer. In: Khosravi-Far R, White E, editors. Programmed cell death in cancer progression and therapy. The Netherlands: Springer; 2006. In press.
42. Nelson D, White E. Exploiting different ways to die. Genes Dev 2004;18:1223–6.

Clinical Cancer Research

A Mouse Model System to Genetically Dissect the Molecular Mechanisms Regulating Tumorigenesis

Kurt Degenhardt and Eileen White

Clin Cancer Res 2006;12:5296-5304.